Catalytic mechanism of scytalone dehydratase from *Magnaporthe grisea*†

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Abstract: The catalytic mechanism of scytalone dehydratase was examined by studying alternative substrates and site-directed mutations of active-site residues. Searches for an enol intermediate by looking for a half-reaction with authentic scytalone and 3,4-dihydro-6,8-dihydroxy-1-(2*H*)-2-[^{13}C]naphthalenone were negative. An alternative substrate, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one (DDBO), was nearly equal to scytalone as substrate for the enzyme, and DDBO's anomeric effect in stabilizing a partial carbocation center at C3 does not substantially contribute to the mechanism. Kinetic analysis of site-directed mutations of active-site amino acid side chains within the enzyme's active site provided an account for the role of these residues in the enzyme-catalyzed dehydration reactions. A concerted E2 elimination for the catalytic mechanism is proposed.

Keywords: scytalone dehydratase; fungicide; fungal melanin; plant disease; enzyme mechanism; X-ray crystallography

1 INTRODUCTION

Scytalone dehydratase (SD, EC 4.2.1.94) is the molecular target of a recently commercialized fungicide, (KTU 3616; 2,2-dichloro-*N*-[1-(4-chlorophenyl)ethyl] - 1 - ethyl - 3 - methylcyclopropanecarboxamide; proposed common name carpropamid), which controls rice blast.[1,2] The enzyme catalyzes the dehydration of two physiological substrates, scytalone and vermelone, in the fungal melanin biosynthetic pathway where 1,3,6,8-tetrahydroxynaphthalene is converted to 1,8-dihydroxynaphthalene through successive reduction and dehydration steps (Fig 1). In *Magnaporthe grisea* (Hebert) Barr, the disease determinant of rice blast, there are direct links between the ability of the organism to produce fungal melanin and its ability to infect rice leaves. Independent genetic knockouts in *M grisea* of SD and trihydroxynaphthalene reductase (3HNR), the target of tricyclazole and other blasticides,[3–11] were non-pathogenic and portrayed phenotypes in culture which revealed accumulation of oxidation products of 1,3,6,8-tetrahydroxynaphthalene and 1,3,8-trihydroxynaphthalene, respectively, and were named rsy (for SD knockouts) and buf (for 3HNR knockouts) to describe the observed coloration of the fungi.[12] The physiological role of fungal melanin in forming an infection organ and in facilitating the invasion of leaf cells has been well characterized.[13]

An X-ray crystal structure of *M grisea* SD complexed with an active site inhibitor was solved and plausible chemical mechanisms for the enzyme-catalyzed dehydration of scytalone were proposed.[14] The absolute stereochemistries of natural substrates, scytalone and vermelone, have been determined so that docking of substrates in the active site could readily be achieved.[15] We examined possible mechanisms for the enzyme catalysis by studying site-directed mutagenesis of active site amino acids and alternative substrates. A summary of our studies and conclusions is presented.

2 MATERIALS AND METHODS

M grisea SD was produced heterologously in *Escherichia coli* Cast & Chalm, and it was purified to homogeneity as judged by SDS-PAGE as described.[16] Purification of site-directed mutants of SD to homogeneity followed very similar protocols. Construction of site-directed mutants and other methods will be described in detail elsewhere.

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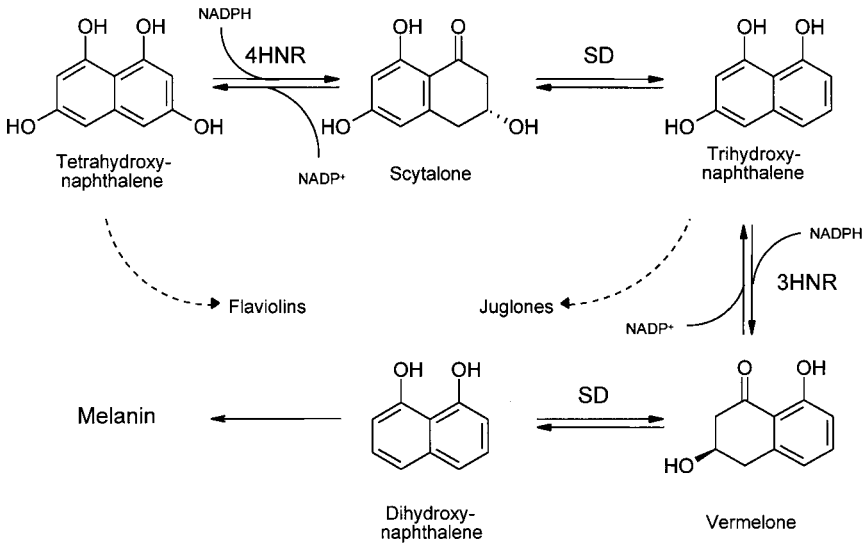

Figure 1. The fungal melanin biosynthetic pathway. 4HNR = tetrahydroxynaphthalene reductase; SD = scytalone dehydratase; 3HNR = trihydroxynaphthalene reductase.

3 RESULTS AND DISCUSSION

Docking of substrate scytalone in the active site of SD[14] to replace the inhibitor in the X-ray structure of SD reaction (see Fig 2) suggested potential scenarios for the enzyme-catalyzed dehydration with the two crystallographic water molecules being significant to the mechanism. The water molecule bound between Y30 and Y50 could provide a proton to the carbonyl of substrate scytalone, promoting enolization. Enolization could be promoted by N131, which is hydrogen-bonded to the substrate's C8 hydroxyl which is, in turn, internally hydrogen-bonded to its own carbonyl. The water molecule bound between H85 and H110 in the inhibitor model could represent the OH leaving group from C3 of substrate.

The amide of the inhibitor in the X-ray structure[14] of SD has sp2 centers which could mimic an enol intermediate in the enzyme-catalyzed transformation of scytalone to 1,3,8-trihydroxynaphthalene. We examined the hypothesis of having a step-wise elimination mechanism with the first step being formation of an enol of scytalone by employing three independent methods. First, we ran the enzyme-catalyzed reaction in deuterated solvent at time points corresponding to varying degrees of completion of the reactions. If the enol intermediate were formed and not fully committed to product formation, it would be expected that some of the recovered scytalone would be deuterated at the C-2 position. Analysis of the reaction mixtures by electrospray ionization mass spectrometry indicated that there was no increase in the mass corresponding to a deuterated scytalone in comparison to authentic scytalone. Second, we prepared, 3,4-dihydro-6,8-dihydroxy-1-(2*H*)-2-[^{13}C]naphthalenone (^{13}C-*des*-hydroxy scytalone, Fig 3) in order to test the half-reaction enolization hypothesis. The ^{13}C material was incubated with SD and analyzed directly by NMR for the formation of an enol of the ^{13}C material by looking for the appropriate spectral shift. There was no indication of the formation of an enol in the ^{13}C material within the active site of SD, even though the small molecule bound tightly to the enzyme, and NMR analysis indicated strong line-broadening indicative of binding. Third, we compared the substrate specificity of SD for scytalone

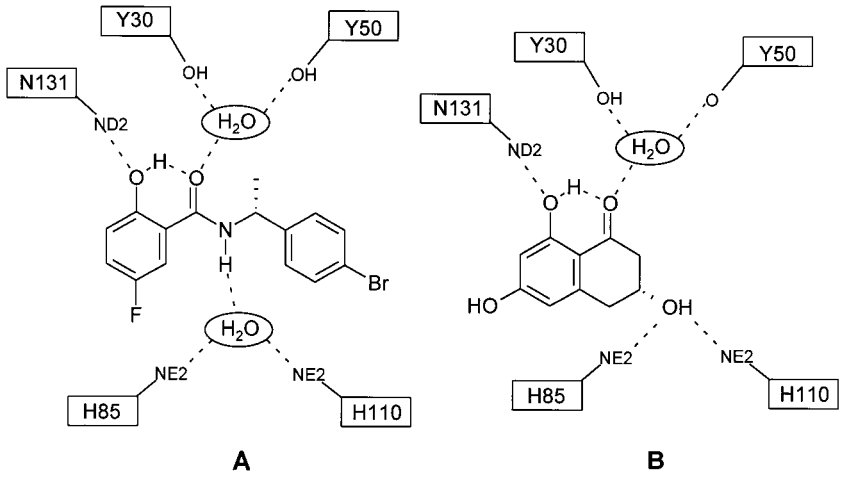

Figure 2. The active site of scytalone dehydratase. (A) With inhibitor bound as reported in the X-ray structure.[14] (B) With substrate scytalone docked in replacing the inhibitor.

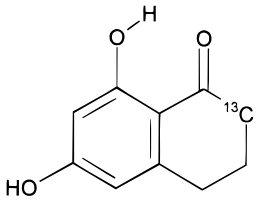

Figure 3. The structure of 3,4-dihydro-6,8-dihydroxy-1-(2*H*)-2-[^{13}C]naphthalenone (^{13}C-*des*-hydroxy scytalone).

versus a non-physiological substrate, 2,3-dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one (DDBO). It was reported that k_{cat} and k_{cat}/K_m values were several-fold larger for the DDBO substrate than for scytalone, implicating loss of hydroxide in a late transition state.[17] However, pre-steady-state single-turnover experiments showed nearly equivalent rates for SD-catalyzed conversions of scytalone and DDBO, challenging the idea of E1 character in the transition state in preference to enol formation either stepwise or in the transition state. The data taken together support a concerted mechanism for the dehydration reactions catalyzed by SD with respect to enolization at C1 and C2 and elimination of the OH from C3 (Fig 4).

Based on the docking of substrate scytalone within the active site of SD, it was anticipated that phenolic OH groups of Y30 and Y50, as well as imadazolyl nitrogens of H85 and H110 side chains, would contribute strongly to catalysis. In addition, from the docking shown in Fig. 2, it was thought that the side chain NH_2 of N131's carboxamide may be important for substrate recognition and in helping to achieve enolization of substrate. These notions were tested through alteration of active-site amino acid side-chains, which was achieved by using site-directed mutagenesis methods. Kinetic constants for substrate scytalone of the wild-type and mutant enzymes were obtained accurately and showed conclusively the importance of the amino acid side-chains in promoting catalysis. Y30F and Y50F mutations decreased k_{cat} by 10-fold and 500-fold, respectively, while K_m values changed little in comparison to wild-type enzyme. H85N and H110N mutations decreased k_{cat} to below measurable levels and 1800-fold, respectively, while the K_m value for H110N increased by a factor of two in comparison to wild-type enzyme. In separate studies it was found that the H85N mutation caused little change in the K_m value for substrate DDBO. In contrast, the N131A mutation imposed a 90-fold decrease in k_{cat} and a 10-fold increase in K_m values. These results suggest

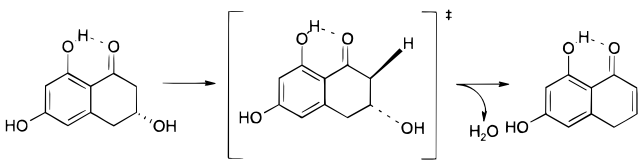

Figure 4. Proposed concerted transition state for the SD-catalyzed dehydration of scytalone.

that Y30, Y50, H85 and H110 side-chain functionalities are involved primarily in promoting the enolization and OH elimination reactions, with little regard to substrate recognition. The N131 carboxamide assists enolization through a strong hydrogen bond to the substrate's C8 OH and thereby is an important factor in substrate recognition. The significance to catalysis of the crystallographic water ligand between Y30 and Y50 has been the subject of a rigorous analysis.[18]

We have developed improved methods for obtaining X-ray quality crystals of SD complexed with a variety of inhibitors, yielding diffraction data sets as high as 1.5 Å. Several SD-inhibitor structures have been solved with these methods, which provide crystals at a pH closer to physiological (pH 7.5 versus pH 5 in the original structure). Recently, Nakasako *et al*[19] solved the X-ray structure of SD complexed with carpropamid by molecular replacement using the SD complex with the salicylamide inhibitor of Fig 2 as the basis. All of these new structures maintain the two crystallographic water molecules within the active site, and dockings of substrate scytalone into the inhibitor binding pockets reaffirm the conclusions we have drawn in this work, mainly that SD catalyzes the dehydration of its substrates *via* a concerted E2 mechanism.

REFERENCES

1 Kurahashi Y, Sakawa S, Kinbara T, Tanaka K and Kagabu S, Biological activity of carpropamid (KTU 3616). A new fungicide for rice blast disease. *Nippon Noyaku Gakkaishi* (*J Pestic Sci*) **22**:108–112 (1997).

2 Tsuji G, Takeda T, Furusawa I, Horino O and Kubo Y, Carpropamid, an anti-rice blast fungicide, inhibits scytalone dehydratase activity and appressorial penetration in *Colletotrichum lagenarium*. *Pestic Biochem Physiol* **57**:211–219 (1997).

3 Andersson A, Jordan D, Schneider G, Valent B and Lindqvist Y, Crystallization and preliminary X-ray diffraction study of 1,3,8-trihydroxynaphthalene reductase from *Magnaporthe grisea*. *Proteins: Structure Function and Genetics* **24**:525–527 (1996).

4 Andersson A, Jordan D, Schneider G and Lindqvist Y, Crystal structure of the ternary complex of 1,3,8-trihydroxynaphthalene reductase from *Magnaporthe grisea* with NADPH and an active-site inhibitor. *Structure* **4**:1161–1170 (1996).

5 Bass RJ, Koch RC, Richards HC and Thorpe JE, Tricyclic amides: a new class of systemic fungicides active against rice blast disease. *J Agric Food Chem* **29**:576–579 (1981).

6 Bell AA and Wheeler MH, Biosynthesis and functions of fungal melanins. *Annu Rev Phytopath* **24**:411–451 (1986).

7 Chida T and Sisler HD, Restoration of appressorial penetration ability by melanin precursors in *Pyricularia oryzae* treated with antipenetrants and in melanin-deficient mutants. *Nippon Noyaku Gakkaishi* (*J Pestic Sci*) **12**:49–55 (1987).

8 Froyd JD, Paget CJ, Guse LR, Dreikorn BA and Pafford JL, Tricyclazole: a new systemic fungicide for control of Pyricularia oryzae on rice. *Phytopathology* **66**:1135–1139 (1976).

9 Thompson JE, Basarab GS, Andersson A, Lindqvist Y and Jordan DB, Trihydroxynaphthalene reductase from *Magnaporthe grisea:* realization of an active center inhibitor and elucidation of the kinetic mechanism. *Biochemistry* **36**:1852–1860 (1997).

10 Wagner K and Scheinpflug H, 4,5,6,7-Tetrachlorophthalide (TCP), a new, highly effective fungicide for the control of *Pyricularia oryzae:* synthesis and biological properties. *Pflanzenschutz-Nachr* (*Am. edn*) **28**:210–217 (1975).
11 Woloshuk CP, Wolkow PM and Sisler HD, The effect of three fungicides, specific for the control of rice blast disease, on the growth and melanin biosynthesis by *Pyricularia oryzae* Cav. *Pestic Sci* **12**:86–90 (1981).
12 Chumley FG and Valent B, Genetic analysis of melanin-deficient, nonpathogenic mutants of *Magnaporthe grisea*. *Molec Plant-Microbe Interactions* **3**:135–143 (1990).
13 Howard RJ and Ferrari MA, Role of melanin in appressorium formation. *Exp Mycol* **13**:403–418 (1989).
14 Lundqvist T, Rice J, Hodge CN, Basarab GS, Pierce J and Lindqvist Y, Crystal structure of scytalone dehydratase – a disease determinant of the rice pathogen, *Magnaporthe grisea*. *Structure* **2**:937–944 (1994).
15 Viviani F, Gaudry M and Marquet A, Deoxygenation of polyphenols by a NADPH-dependent reductase. Syntheses and absolute configurations of (+)-scytalone and (−)-vermelone. *New J Chem* **16**:81–87 (1992).
16 Lundqvist T, Weber PC, Hodge CN, Braswell EH, Rice J and Pierce J, Preliminary crystallographic studies on scytalone dehydratase from *Magnaporthe grisea*. *J Mol Biol* **232**:999–1002 (1993).
17 Thompson JE, Basarab GS, Pierce J, Hodge CN and Jordan DB 2,3-Dihydro-2,5-dihydroxy-4*H*-benzopyran-4-one: a non-physiological substrate for fungal melanin biosynthetic enzymes. *Anal Biochem* **255**:1–6 (1998).
18 Zheng Y-J and Bruice TJ, Role of a critical water in scytalone dehydratase catalyzed reaction. *Proc Natl Acad Sci USA* **95**:4158–4163 (1998).
19 Nakasako M, Motoyama T, Kurahashi Y and Yamaguchi I, Cryogenic X-ray crystal analysis for the complex of scytalone dehydratase of a rice blast fungus and its tight-binding inhibitor, carpropamid: The structural basis of tight-binding inhibition. *Biochemistry* **37**:9931–9939 (1998).